United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 608

Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serovar *pomona* Bacterins

Date: **August 8, 2006**

Number: SAM 608.02

Supersedes: BBSAM0608.01, July 1, 1999

Standard Requirement: 9 CFR, Part 113.101

Contact: Mary C. Rasmusson, (515) 663-7831

Approvals:

/s/Paul J. Hauer Date: 29Aug06
Paul J. Hauer, Section Leader
Bacteriology

/s/Byron E. Rippke Date: 31Aug06
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 01Sep06
Rebecca L.W. Hyde, Section Leader
Quality Assurance
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serovar *pomona* Bacterins

Table of Contents

Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serovar *pomona* Bacterins

1. Introduction

This Supplemental Assay Method (SAM) describes the hamster vaccination-challenge method used to determine potency of *Leptospira pomona* bacterins as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.101.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Microscope with darkfield capability

2.1.2 Forceps, 5 1/2-inch, rat-tooth

2.1.3 Dissecting pins, 1- to 1 1/2-inch

2.1.4 Necropsy board

2.1.5 Stomacher® blender and sterile bags (alternatively, tissue grinders, 15-mL, TenBroeck, may be used)

2.1.6 Balance, analytical

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Syringes, 1-mL tuberculin

2.2.2 Needles, appropriate size

2.2.3 Scalpels

2.2.4 Glass screw-top tubes, 20 x 150-mm (or equivalent container)

2.2.5 Serum bottles with rubber stoppers (or equivalent container for preparing dilutions of liver homogenate)

2.2.6 70% (v/v) ethanol

2.2.7 Microscope slides and cover slips

2.2.8 Pipettes, assorted sizes, cotton-plugged

2.2.9 1% Bovine Serum Albumin Diluent (BSAD)

2.2.10 P80-BA semi-solid medium

2.2.11 0.85% NaCl solution (saline)

2.2.12 *L. pomona* challenge culture, hamster-virulent

2.3 Animals

2.3.1 Hamsters, adult, 50-90 g. Ten hamsters are required for each bacterin tested. Thirty additional hamsters are required per test session for nonvaccinated controls and LD_{50} titration.

2.3.2 The hamsters must be obtained from the same source and colony. Use either all male or all female hamsters for any one test.

2.3.3 House and feed all hamsters in an identical manner.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in the safe handling of live *Leptospira* spp. Personnel need specific training in the care and handling of laboratory hamsters and in the performance of this assay.

3.2 Preparation of equipment and supplies

3.2.1 Operate and maintain all equipment according to manufacturers' recommendations and applicable in-house standard operating procedures.

3.2.2 Sterilize all glassware before use.

3.2.3 Use only sterile supplies (pipettes, syringes, needles, rubber stoppers, etc.).

3.3 Preparation of reagents

3.3.1 1% Bovine Serum Albumin Diluent--National Veterinary Services Laboratories (NVSL) Media #20133

Sodium phosphate, dibasic	0.664 g
Potassium phosphate, monobasic	0.087 g
Bovine serum albumin, fraction V	10 g
Deionized water	q.s. 1.0 L

Mix until dissolved. If necessary, adjust pH to 7.5. Sterilize by filtration, using a 0.22-µm filter. Store at 20°- 25°C for no longer than 1 year.

3.3.2 0.85% saline--NVSL Media #30201

Sodium chloride	8.5 g
Deionized water	q.s. 1.0 L

Autoclave at 121°- 125°C for 15 to 20 minutes. Store at 20°- 25°C for no longer than 1 year.

3.3.3 P80-BA semi-solid medium--NVSL Media #10117

Sodium phosphate, dibasic	0.664 g
Potassium phosphate, monobasic	0.087 g
Sodium chloride	1.925 g
Ammonium chloride	0.268 g
Magnesium chloride	0.191 g
Deionized water	790 mL

Stir to dissolve. Add:

Cupric sulfate solution (300 mg/L, pH 5.8)	1 mL
Zinc sulfate solution (0.4g/L, pH 6.3)	10 mL
Ferrous sulfate solution (2.5 g/L)	20 mL
L-cystine	0.2 g

Stir. Do not attempt to dissolve L-cystine completely. Do not heat.
Filter through triple thickness #1 Whatman paper. If filtrate is not clear, filter again.

Combine filtered media with:

Vitamin B12 solution (10 mg/L)	20 mL
Thiamine HCl solution (2 g/L, pH 3.8)	0.1 mL
Tween 80	1.2 mL
Deionized water	q.s. 1 L

Place 800 mL of this mixture in a large container and add 1.3 g purified agar. Autoclave at 121°- 125°C for 20 to 25 minutes. Cool to 56°±1°C.

Combine the following:

Bovine serum albumin, fraction V	20 g
Sodium phosphate, dibasic	0.133 g
Potassium phosphate, monobasic	0.017 g
Deionized water	q.s. 200 mL

Adjust pH to 7.4 to 7.6 and sterilize by filtration (0.2 µm).

Add filtered albumin solution to the cooled (56°C) solution prepared previously.

Adjust to pH 7.2 to 7.8 with sterile 10% NaOH. Dispense in 9 mL aliquots into screw-capped tubes. Store, tightly capped at 20°- 25°C for no longer than 1 year.

3.4 Preparation of the sample

3.4.1 Shake each bacterin to mix contents thoroughly.

3.4.2 Disinfect the top of the bacterin container with 70% ethanol.

3.4.3 Dilute each bacterin with saline so that 1 hamster dose (0.25 mL) is equivalent to 1/800 of the recommended host-animal dose.

1. For 2-mL-dose products, dilute the bacterin 1:100 in saline.

2. For 5-mL-dose products, dilute the bacterin 1:40 in saline.

4. Performance of the test

4.1 Vaccination of hamsters

4.1.1 For each bacterin to be tested, vaccinate 10 hamsters with 0.25 mL of appropriately diluted bacterin (see **Section 3.4.3**) using the route recommended by the manufacturer. If the recommended vaccination route is intramuscular, or if the product is labeled for either intramuscular or subcutaneous use, vaccinate the hamsters intramuscularly in the hind leg. If the label limits administration of that product to the subcutaneous route, vaccinate the hamsters subcutaneously in the abdominal area. For all vaccinations, use a 1.0-mL syringe fitted with an appropriate size needle.

4.1.2 Retain 10 nonvaccinated hamsters as controls.

4.1.3 Retain 20 nonvaccinated hamsters to determine the LD_{50} of the challenge inoculum.

4.1.4 Challenge all hamsters with a virulent suspension of *L. pomona* 14 to 18 days after vaccination.

4.2 Challenge procedure

The challenge inoculum is a liver homogenate from a clinically ill hamster. The Center for Veterinary Biologics (CVB) maintains virulent challenge organisms by serial passage through hamsters on a routine basis.

4.2.1 Select a clinically ill (preferably moribund) hamster from a group of hamsters that were infected 3 to 4 days previously with *L. pomona*.

4.2.2 Euthanize the hamster with CO_2. Rapid administration from a compressed gas cylinder is recommended because the inflow to the chamber can be regulated precisely. Follow the euthanasia procedure approved by the Animal Care and Use Committee.

4.2.3 Pin the dead hamster to a posting board (ventral aspect up), and disinfect the skin with 70% ethanol.

4.2.4 Using aseptic technique, reflect the abdominal skin. Reflect the abdominal musculature to expose the abdominal viscera. Discard the instruments used to open the abdomen.

4.2.5 Using fresh instruments, aseptically remove approximately 1 gram of liver tissue. Place liver in a sterile, previously weighed container. Weigh the container

again with liver tissue and add or subtract additional tissue to obtain a mass as close to 1.0 gram as possible ± 0.1 gram. Aseptically place the liver in a sterile blender bag. Add 9 mL of sterile BSAD to the bag. Thoroughly homogenize the liver, taking care to avoid foam formation. This suspension is considered the 1:10 dilution.

4.2.6 Prepare 5 additional serial tenfold dilutions (10^{-2} through 10^{-6}) of the tissue suspension in BSAD (1.0 mL suspension + 9.0 mL diluent). Hold the dilutions at room temperature (20°- 25°C) and complete challenge inoculations within 1 hour after preparation of dilutions.

4.2.7 Place 2 drops of the 10^{-4} dilution on a microscope slide, cover with a coverslip, and examine under a 200X magnification with a darkfield microscope. The 10^{-4} dilution should have approximately 4 to 20 organisms per field.

4.2.8. If the 10^{-4} dilution has 4 to 20 organisms per field, use the 10^{-6} dilution for the challenge inoculum. (This usually is sufficient to deliver the required challenge of 10-10,000 LD_{50}.) The challenge inoculum should be prepared in an appropriate sterile container.

Note: If the 10^{-4} dilution contains >20 organisms per field, dilute the challenge with BSAD until numbers of organisms are between 4 and 20 per field. If <4 organisms are present per field in the 10^{-4} dilution, examine the 10^{-3} dilution; if the 10^{-3} dilution contains the required number of organisms, challenge with the 10^{-5} dilution. If the 10^{-3} dilution contains <4 organisms per field, select another clinically ill hamster (Section 4.2.1) and prepare another challenge inoculum that more closely matches the desired organism density.

4.2.9 Prepare 4 additional tenfold dilutions beyond the dilution selected for the challenge inoculum. Retain these dilutions to determine the LD_{50} of the challenge inoculum.

4.2.10 The remaining liver homogenate from the challenge preparation may be used to infect additional hamsters to serve as a source of inoculum for future potency tests. The dilution and dose volume should be tailored to suit the frequency with which serial passage is performed.

4.2.11 Transfer 1 mL of the challenge inoculum into 9 mL of P80-BA semisolid medium. Incubate semisolid cultures at 25°- 30° C for 2 to 4 weeks. Cultures may be maintained at 20°- 25°C for up to 6 months. These cultures serve as an archival source of the challenge inoculum if additional analysis is warranted. The CVB prepares additional cultures (i.e., duplicate cultures of inoculum from each of the 10^{-4} through 10^{-10} dilutions) for internal use.

4.3 Challenge of test hamsters

4.3.1 Within 1 hour after preparation, inject intraperitoneally (IP) 0.2 mL of the dilution selected in **Section 4.2.8** into each of the vaccinated hamsters and 10 nonvaccinated control hamsters. Use a 1.0-mL syringe fitted with an appropriate size needle.

4.3.2 Inject 5 hamsters (0.2 mL, IP) with each of the dilutions prepared in **Section 4.2.9**. These 4 groups of hamsters will be used to calculate the LD_{50} of the challenge.

4.3.3 Disinfect all work surfaces with 70% ethanol. Sterilize all contaminated equipment and supplies in the autoclave.

4.4 Observation of hamsters after challenge

4.4.1 Observe all hamsters daily for 14 days following challenge. Record deaths.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

4.4.2 At the end of the 14-day observation period, count the remaining hamsters and record results.

4.4.3 Calculate the LD_{50} of the challenge inoculum using the Reed-Muench or Spearman-Kärber method of calculation.

5. Interpretation of the test results

5.1 Interpret the results as described in 9 CFR, Part 113.101.

5.2 If 8 or more nonvaccinated controls die and the hamsters received a challenge of 10-10,000 LD_{50}, the test is valid.

5.3 If 3 or 4 vaccinates die in the first stage test, conduct a second stage test in a manner identical to the first stage. If the second stage is used, evaluate each serial according to the second part of the table. Serials pass or fail on the basis of cumulative results. Evaluate the results according to the following table.

Stage	Number of Vaccinates	Cumulative Number of Vaccinates	Cumulative Total Dead Hamsters for Satisfactory Serial	Cumulative Total Dead Hamsters for Unsatisfactory Serial
1	10	10	2 or less	5 or more
2	10	20	5 or less	6 or more

6. Report of test results

Report the results of the test(s) as described by standard operating procedures..

7. References

Code of Federal Regulations, Title 9, Part 113.101, U.S. Government Printing Office, Washington, DC, 2006.

8. Summary of revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.1** A Stomacher® blender and analytical balance have been added to the list of equipment and instrumentation.
- **2.2** The list of reagents and supplies has been updated to more accurately reflect what is used for this assay method.
- **3.3** The formulas have been updated to reflect that the solution may be stored at room temperature for up to one year.
- **3.3.3** P80-BA semi-solid medium has been added to the Preparation of Reagents section.
- References to internal CVB documents have been replaced with summary information.
- The contact person has been changed to Mary C. Rasmusson.